

IMMUNOMODULATORY ACTIVITY OF METHANOLIC EXTRACT OF *MORUS ALBA* LINN. (MULBERRY) LEAVES

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ABSTRACT

The leaves of *Morus alba* Linn. (Family: Moraceae) commonly known as mulberry are mainly used as food for the silkworms and they are sometimes eaten as vegetable or used as cattle fodder in different parts of the world. The effect of *Morus alba* on the immune system was evaluated by using different experimental models such as carbon clearance test, cyclophosphamide induced neutropenia, neutrophil adhesion test, effect on serum immunoglobulins, mice lethality test and indirect haemagglutination test. Methanolic extract of *Morus alba* was administered orally at low dose and high dose of 100 mg/kg and 1 g/kg respectively and *Ocimum sanctum* (100 mg/kg, po) was used as standard drug. *Morus alba* extract in both doses increased the levels of serum immunoglobulins and prevented the mortality induced by bovine *Pasteurella multocida* in mice. It also increased the circulating antibody titre in indirect haemagglutination test. On the other hand, it showed significant increase in the phagocytic index in carbon clearance assay, a significant protection against cyclophosphamide induced neutropenia and increased the adhesion of neutrophils in the neutrophil adhesion test. Hence, it was concluded that *Morus alba* increases both humoral immunity and cell mediated immunity.

Keywords: *Morus alba* Linn, humoral immunity, serum immunoglobulin, phagocytic index, cell mediated immunity.

INTRODUCTION

The leaves of *Morus alba* Linn. (Family: Moraceae) are used as food for the silkworms, as vegetable and as cattle fodder in different parts of the World. Apart from its use as vegetable, the leaves of the plant are used for the treatment of a variety of disorders traditionally. The medicinal properties attributed to mulberry are extensive. Topically, it is applied for the treatment of wounds (Duke and Wain, 1981). Internally, it is used to relieve insomnia, regulate bleeding during menstruation, treat digestive disturbances, to ease cough and asthmatic breathing, reduce fever and inflammation, as hypolipidemic, antiageing, antifilaria, diuretic, and antiulcer agent (Kimura and Hiromichi, 1986; Him-Che, 1985; Ouyang, 2006).

Mulberry is known to possess hypoglycemic (Lemus *et al.*, 1999; Taniguchi *et al.*, 1998; Chen *et al.*, 1995; Hansawasdi and Kawabata, 2006), antibacterial (Bown, 1995), astringent (Duke and Ayensu, 1985), diaphoretic (Bown, 1995), antiviral (Nam *et al.*, 2002) and anticancer (Nam *et al.*, 2002) effects among a wide range of pharmacological activities reported for this plant. It is believed in the traditional medicine that mulberry leaves possess immunomodulatory activity. Furthermore, the polysaccharide isolated from mulberry is reported to have immunomodulatory activity (Kim *et al.*, 2000). The 1-

deoxynojirimycin present in leaves, roots and seeds is known to be effective in the treatment of AIDS (Sergio, 1989; Oku *et al.*, 2006). On the contrary, interaction study of morin isolated from mulberry with cyclosporine reported that morin maintained the immunosuppression induced by cyclosporine and also decreased the production of nitric oxide from the macrophages (Fang *et al.*, 2005).

The present study was undertaken to evaluate the effect of methanolic extract of *Morus alba* Linn. leaves on the immune system using different experimental models to substantiate the traditional claim. The study helps in understanding the effect of *Morus alba* leaves on different components on the immune system.

MATERIALS AND METHODS

Experimental animals

Albino Wistar rats weighing between 200-250 gm and Swiss albino mice weighing between 25-35 gm were used. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control, and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food (Lipton India Ltd., Mumbai, India) and water *ad libitum*.

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Procurement of plant material and extraction

Mulberry leaves were purchased from local market. The plant was identified and authenticated by Regional Research Institute (Bangalore, India) and a voucher specimen (RRI/ BNG/ SMP/ Drug Authentication/ 2007-08/ 277) is deposited in the institute for future reference. The leaves were shade dried and extracted using methanol in a soxhlet. The ethanolic extract of *Ocimum sanctum* was used as standard immunomodulatory agent.

Chemicals

Leishmann's stain, Indian ink and gluteraldehyde were purchased from Merck (Mumbai, India). WBC diluting fluid, zinc sulphate and barium chloride were from Nice Chemicals (Cochin, India). Cyclophosphamide (Endoxan Injection) was from German Remedies (Mumbai, India).

Pasteurella multocida of bovine origin and its vaccine were obtained from Institute of Animal Health and Veterinary Biologicals (Bangalore, India).

Acute toxicity studies

(<http://www.epa.gov/oppts/home/guideline.htm>)

The acute toxicity study was carried out according to the limit test described in the OPPTS guidelines. Briefly, a test dose of 2 g/kg and 5 g/kg were given orally to the mice. The extract was found to be safe at the dose of 5 g/kg, po. Hence, 1/50th and 1/5th of the safe dose corresponding to 100 mg/kg and 1 g/kg orally were selected as low and high dose respectively.

Selection of Dose and Treatment period

The animals were divided into three groups consisting of six animals each. The first group served as control (vehicle 1 ml/100 g, po), the second group received the ethanolic extract of *Ocimum sanctum* (OSE) at a dose of (100 mg/kg, po) (Sharma *et al.*, 2001). The third and fourth group received the low dose (100 mg/kg, po) of *Morus alba* extract (MALD) and high dose (1 g/kg, po) of *Morus alba* extract (MAHD) respectively. Mice lethality test had five groups, out of which two served as controls, one positive control and the other negative control. The drug solutions were prepared in distilled water and were administered orally.

Neutrophil Adhesion test (Fulzele *et al.*, 2003; Shinde *et al.*, 1999)

The rats were treated orally with vehicle or extracts for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinised vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 min at 37 °C. The incubated blood samples were again analyzed for DLC. The percentage of neutrophils in the treated and untreated blood was determined and the difference was taken as index of neutrophil adhesion.

Mice lethality test (Ramanatha *et al.*, 1995)

Swiss albino mice were treated with different extracts or vehicle orally for 21 days. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine). On the 21st day, the animals were challenged subcutaneously with 0.2 ml of lethal dose (25 x LD₅₀) of *Pasteurella multocida* (bovine origin) containing 10⁷ cells per ml. The animals were observed for a period of 72 hr and the mortality ratio was determined using the formula:

$$\text{Mortality ratio} : \frac{\text{Number of animals dead}}{\text{Total number of animals}}$$

Cyclophosphamide induced neutropenia (Thatte *et al.*, 1987)

Swiss albino mice received the drug or vehicle orally for 10 days. On 10th day, neutropenic dose of cyclophosphamide (200 mg/kg, sc) was injected and this day was labeled as day zero. Blood was collected, the total leukocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of the control group.

Carbon clearance test (Jayathirtha and Mishra, 2004; Gokhale *et al.*, 2003)

Swiss albino mice were treated with the drug or vehicle orally for 5 days. After 48 hr of the last dose of the drug, animals were injected 0.1 ml of Indian ink via the tail vein. Blood samples were withdrawn at 0 and 15 min after injection. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following equation:

$$K = (\text{Log}_e \text{OD1} - \text{Log}_e \text{OD2})/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively.

Effect on serum immunoglobulins

The drugs were administered to female albino rats orally for 21 days. Six hours after the last dose of drug, blood was collected and the serum was used for estimation of immunoglobulin levels using method devised by Mullen (1975).

Briefly, for each serum sample to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution were prepared. To each, 0.1 ml of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained

(sample-blank) was compared with that obtained with standard barium sulphate (BaSO_4) solution. The standard BaSO_4 solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acid. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

Indirect Haemagglutination Test (IHA test) (Fulzele et al., 2003)

Rats were pretreated with the drugs for 14 days and each rat was immunized with 0.5×10^9 sheep red blood cells (SRBCs) intraperitoneally, including control rats. The day of immunization was referred to as day 0. The drug treatment was continued for another 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions with SRBC (0.025×10^9 cells) in microtitre plates. The plates were incubated at room temperature for 2 hr and examined visually for agglutination. The minimum volume of serum showing haemagglutination was expressed as haemagglutination (HA) titre.

STATISTICAL ANALYSIS

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's comparison test. The values are expressed as mean $\pm$ SEM and $P < 0.05$ was considered significant.

RESULTS

Effect on neutrophil adhesion test

Incubation of neutrophils with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. Both doses of *Morus alba* extract and OSE showed significant increase in the neutrophil adhesion when compared to control. The MALD was significantly more effective compared to MAHD. The neutrophil count in untreated blood was also increased by all the treatments (fig. 1).

Effect on Mice Lethality Test

Administration of *Pasteurella multocida* to control animals produced 100% mortality within 72 hr of administration. One out of six animals that received vaccination tolerated the lethal dose of the organism and the mortality was 83.33%. The MALD and MAHD reduced the mortality ratio to 33.33% and 50% respectively and OSE showed 33.33% decrease in the mortality ratio when compared to control (table 1).

Effect on cyclophosphamide induced neutropenia

The neutropenic dose of cyclophosphamide reduced the TLC in control animals by 57.87%. Administration of *Morus alba* for 10 days before cyclophosphamide administration at low dose (MALD) produced 30.99%

and high dose (MAHD) produced 51% reduction in TLC, while OSE pretreatment produced 38.64% decrease in TLC when compared to initial values. The neutrophil count (%) was reduced by 75.71% in cyclophosphamide treated control, 13.71% in MALD, 59.14% in MAHD pretreated animals and 9.03% in OSE pre treated animals when compared to initial values (table 2).

Effect on carbon clearance test

Both the doses of *Morus alba* extract and OSE showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs (table 3).

Effect on serum immunoglobulins

Both the doses of *Morus alba* extract and OSE showed a significant increase in the serum immunoglobulin levels when compared to control (table 3).

Effect on indirect haemagglutination test

The haemagglutinating antibody (HA) titer value was significantly increased in animals that received vaccination along with *Morus alba* extract at two different doses or OSE compared to animals that received vaccination alone (table 3).

DISCUSSION

The results of the present study suggest that *Morus alba* extract affects humoral immunity as shown by its effect in the indirect haemagglutination test, serum immunoglobulin levels and mice lethality test and it also has effect on the cell mediated immunity as it showed significant increase in the neutrophil adhesion, carbon clearance and a reduction in cyclophosphamide induced neutropenia.

The adhesion of neutrophils to nylon fibres describes the margination of cells in the blood vessels and the number of neutrophils reaching the site of inflammation (Shinde et al., 1999). *Morus alba* extract at both doses showed a significant increase in the neutrophil adhesion to nylon fibres. This might be due to the upregulation of the $\beta 2$ integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres (Srikumar et al., 2005). Hence, it was inferred that *Morus alba* extract causes stimulation of neutrophils towards the site of inflammation.

The mouse lethality test is one of the widely used tests to evaluate serological responses in animals immunized with vaccines. *Pasteurella multocida* is pathogenic to mice. The mouse lethality test involves injecting mice with the vaccine prior to the administration of the bacterial culture and determining the mortality ratio (Finco et al., 2001). The vaccination will cause production of antibodies. If the

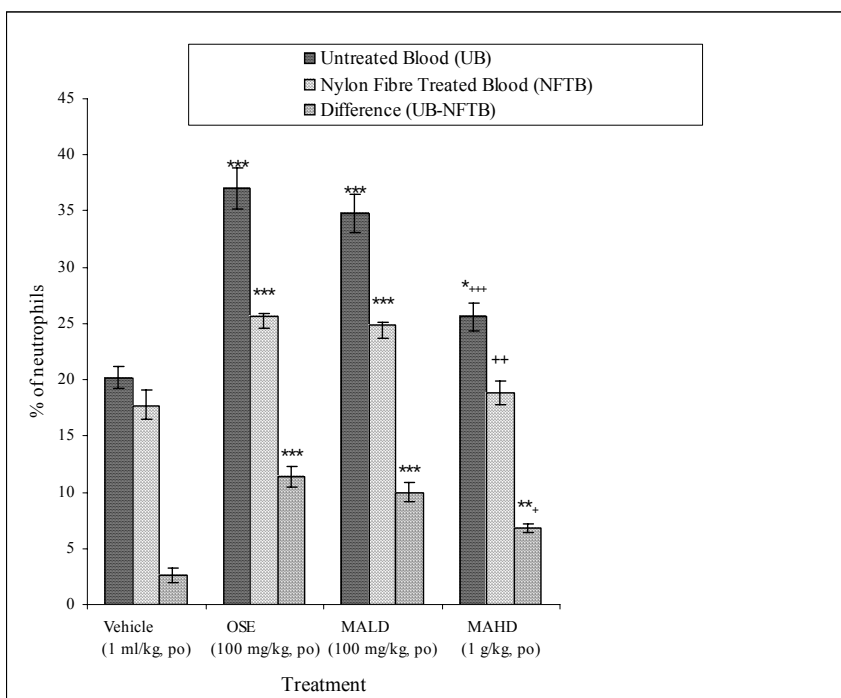


Fig. 1: Effect on neutrophils adhesion in rats.

All Values are expressed as mean $\pm$ SEM of six observations. * P <0.05, ** P <0.01, *** P <0.001 when compared to control, P <0.05, ++ P <0.01, +++ P <0.001 when compared to low dose.

Table 1: Effect on mice lethality test.

Groups	Day-1	Day-2	Day-3	Mortality ratio
No Drug - No Vaccination	4	2	-	100%
No Drug Vaccination	-	2	3	83.33%
OSE, Vaccination	-	1	1	33.33%
MALD, Vaccination	-	1	1	33.33%
MAHD, Vaccination	-	1	2	50%

n=6

drug has an ability to enhance the production of antibodies to such an extent that antibodies produced can counter the pathogen, then the animals survive. *Morus alba* extract at low dose showed 33.33% and high dose showed 50% reduction in the mortality ratio when compared to control.

The cyclophosphamide induced neutropenia model concentrates on the effect of drugs on the haemopoietic system (Diwanay *et al.*, 2004). *Morus alba* extract at low dose and high dose caused 13.71% and 59.14% decrease in the cyclophosphamide induced neutropenia suggesting that it attenuates the effect of cyclophosphamide on the haemopoietic system.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play important role in the clearance of particles from the

bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Gokhale *et al.*, 2003). *Morus alba* extract at both doses and OSE showed significant increase in the phagocytic index. Hence, these agents may increase the activity of the reticuloendothelial system.

The estimation of serum immunoglobulin levels was used to evaluate the increase in serum immunoglobulin production after the administration of the drugs. Immunoglobulins are antibodies that react specifically with the antigen. The zinc sulphate turbidity test is used to gain a rough estimation of the amount of immunoglobulins present in the serum. Zinc sulphate causes precipitation of the immunoglobulins making the solution cloudy. A lack of cloudiness signifies lack of immunoglobulins (Llamapaedia, 2005). The turbidity is expressed as ZST units, which in turn indicate the amount

Table 2: Effect on cyclophosphamide induced neutropenia.

Treatment	Reduction in no. of leucocytes after cyclophosphamide treatment	% reduction compared to initial value	Reduction in neutrophil count (%) compared to initial values	% reduction compared to initial value
Vehicle (1 ml/kg, po)	3030 ± 111.36	57.87	16.2 ± 0.7348	75.71
OSE (100 mg/kg, po)	2260 ± 88.60***	38.64	02.4 ± 0.2449***	9.03
MALD (100 mg/kg, po)	2110 ± 92.73***	30.99	03.4 ± 0.2449***	13.71
MAHD (1 g/kg, po)	2550 ± 72.45* ⁺	51.00	13.6 ± 0.6782* ⁺⁺⁺	59.14

All values are mean ± SEM, n = 6, ***P<0.001, **P<0.01, *P<0.01 when compared to control group, ⁺P<0.05, ⁺⁺⁺P<0.001, when compared to low dose.

Table 3: Effect on phagocytic index in carbon clearance assay, serum immunoglobulin levels and (HA titre) in IHA test.

Treatment	Phagocytic index	Serum immunoglobulin level (ZST units)	HA titre
Vehicle (1 ml/kg, po)	0.0050 ± 0.0001	23.265 ± 0.3896	0.106700 ± 0.033700
OSE (100 mg/kg, po)	0.0192 ± 0.0008***	35.585 ± 0.7734***	0.000007 ± 0.000001**
MALD (100 mg/kg, po)	0.0165 ± 0.0021***	30.057 ± 0.6115***	0.000036 ± 0.000095**
MAHD (1 g/kg, po)	0.0132 ± 0.0010** ^{ns}	26.746 ± 0.5123** ⁺⁺	0.004200 ± 0.001300** ^{ns}

All values are mean ± SEM, n = 6, ***P<0.001, **P<0.01 when compared to control group, ^{ns}P>0.05 ⁺⁺P<0.01, when compared to low dose.

of immunoglobulin present in the sample. *Morus alba* extract at both the doses showed a significant increase in the serum immunoglobulin levels.

The indirect haemagglutination test was performed to confirm the effect of *Morus alba* extract on the humoral arm of the immune system. The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross linking to form latex that are more readily ingested by phagocytic cells (Gokhale *et al.*, 2003). The results showed that levels of circulating antibodies are increased if the test animals are pretreated with *Morus alba* extract or OSE.

The mulberry leaves contains quercetin 3-glucoside (isoquercitrin), morin, 1-deoxynojirimycin, kaempferol 3-glucoside (astragalin), carotene, vitamin B₁, folic acid, folinic acid and vitamin D. The presence of glutathione in leaves has been reported (Jayal and Kehar, 1962; Rao *et al.*, 1971). The exact constituent responsible for the immunomodulatory effect is not known. However, of these constituents, only folic acid is known to increase immune response in elderly (VitaNet® Staff, 2008). On the contrary, morin is reported to maintain immunosuppression induced by cyclosporine and also decreased the production of nitric oxide from the macrophages (Fang *et al.*, 2005). However, it is also worth mentioning that 1-deoxynojirimycin is an alpha-glucosidase I inhibitor that inhibits human

immunodeficiency virus (HIV)-1 replication by altering glycosylation of gp120 (Sergio, 1989; Oku *et al.*, 2006). The present data is insufficient to predict the exact constituent(s) responsible for immunomodulatory activity and further work has to be carried out to determine the constituent(s) responsible for the effect.

To conclude, the extract of *Morus alba* has a significant effect on the humoral and cell mediated immunity in experimental animals.

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